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Differential effects of exercise intensities in hippocampal BDNF, inflammatory cytokines and cell proliferation in rats during the postnatal brain development

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HIGHLIGHTS

- BDNF levels and cellular proliferation in the hippocampus are dependents of exercise intensity.
- It was noted that exercise intensity is an inflammation-inducing factor.
- Exercise-induced inflammatory response is also related to developmental stage.

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ABSTRACT

It has been established that low intensities of exercise produce beneficial effects for the brain, while high intensities can cause some neuronal damage (e.g. exacerbated inflammatory response and cell death). Although these effects are documented in the mature brain, the influence of exercise intensities in the developing brain has been poorly explored. To investigate the impact of exercise intensity in developing rats, we evaluated the hippocampal level of brain derived neurotrophic factor (BDNF), inflammatory cytokines (TNFα, IL6 and IL10) and the occurrence of hippocampal cell degeneration and proliferation at different stages of postnatal brain development of rats submitted to two physical exercise intensities. To this point, male rats were divided into different age groups: P21, P31, P41 and P51. Each age group was submitted to two exercise intensities (low and high) on a treadmill over 10 consecutive days, except the control rats. We verified that the density of proliferating cells was significantly higher in the dentate gyrus of rats submitted to low-intensity exercise from P21 to P30 compared with high-intensity exercise and control rats. A significant increase of proliferative cell density was found in rats submitted to high-intensity exercise from P31 to P40 when compared to low-intensity exercise and control rats. Elevated hippocampal levels of IL6 were detected in rats submitted to high-intensity exercise from P21 to P30 compared to control rats. From P41 to P50 period, higher levels of BDNF, TNFα and IL10 were found in the hippocampal formation of rats submitted to high-intensity exercise in relation to their control rats. Our data show that exercise-induced neuroplastic effects on BDNF levels and cellular proliferation in the hippocampal region are dependent on exercise intensity and developmental period. Thus, exercise intensity is an inflammation-inducing factor and exercise-induced inflammatory response during the postnatal brain development is also related to developmental stage. Our findings indicate that neuroplastic changes induced by exercise in developing rats depend on both age and training intensity.

1. Introduction

Brain development is a complex process characterized by a series of critical stages. Each stage must be properly fulfilled in order for the brain to configure their normal structure. Although the brain's structure is formed before birth, its full development depends on postnatal stimuli from the environment. Stimuli during

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this postnatal period may modulate the brain's functional maturation and determine its lifelong integrity [1]. Evidence from human studies has shown that some environmental stimuli such as physical activity habits in infancy and adolescence seem to have a favorable influence [3,11,22]. For instance, a positive correlation between physical activity and learning and intelligence scores has been observed in school-age children [22]. In preadolescent children, single or various exercise sessions may modify neuronal activity, improve the performance in attention tasks, and enhance reading comprehension and response accuracy in academic achievement tests [3,11]. Data from animal studies have shown that exercise can modulate the functional maturation of the developing brain by means of neuroplastic processes [8,24]. In a study conducted by our group, we verified that an exercise program undertaken during adolescent period of rats was able to increase axonal density of granule cells of the dentate gyrus, to enhance hippocampal expression of neurotrophic factors, and to improve learning and memory [8]. Together, these interesting data suggest that physical exercise during postnatal development results in positive changes for the brain, particularly in the hippocampal formation – a highly plastic region of the brain linked to cognitive and emotional processes. However, it is important to point out that exercise-induced neuroplastic effects may vary according to animal age [14] and training intensity [15].

It is usually accepted that low and moderate intensities of exercise produce beneficial effects for the brain, while high intensities can cause some damage (e.g. cell death). Indeed, harmful effects could appear in undue conditions of physical or psychological stress. Of the few studies conducted with physical training in juvenile rats, it was demonstrated that exercise-induced neuroplasticity was dependent upon exercise intensity [15]. The low-, but not the high-, intensity exercise resulted in enhanced neurogenesis and gene expression of brain derived neurotrophic factor (BDNF), *N*-methyl-D-aspartate receptor type 1 (NMDAR1) and fetal liver kinase-1 (Flk-1) in the hippocampal formation of 35-day-old rats [15]. Nevertheless, it is unclear whether there is a developmental stage in which exercise intensity could induce a greater neuroplastic influence. Moreover, it has been described that exercise can alter the brain expression of inflammatory cytokines [4,5], small cell-signaling proteins known to affect the integrity of the blood–brain barrier and induce cell death during development [6,10]. In view of these observations, we evaluated the hippocampal levels of BDNF and inflammatory cytokines and the occurrence of hippocampal cell degeneration and proliferation at different stages of postnatal brain development (prepuberte: P21–P30; juvenile: P31–P40; adolescent: P41–P50; late adolescent: P51–P60) of rats submitted to two physical exercise intensities.

2. Methods

2.1. Exercise paradigm

Wistar rats were used in this study. All experimental protocols described below were approved by the ethics committee of the Universidade Federal de São Paulo (#0607/09). The colony room was maintained at 21 ± 2 °C with a 12 h light/dark schedule, and ad libitum food and water throughout the experiments. Male rats were divided into different age groups: P21, P31, P41 and P51 ($n = 44$ in each group). Each age group was submitted to two exercise intensities (low and high) on a treadmill (Columbus instruments) over 10 consecutive days, except the control rats. Exercise intensities were determined in accordance with the development stage of each group: the rats ran from P21 to P30 at low-intensity of 12 m/min and high-intensity of 16 m/min, from P31 to P40 at low-intensity of 14 m/min and high-intensity of 18 m/min, from P41 to P50 at low-intensity of 16 m/min and high-intensity of 20 m/min, and from P51 to P60 at low-intensity of 18 m/min and high-intensity of 22 m/min. Rats submitted to low- and high-intensity exercise ran on a treadmill for 30 min per day. Exercise sessions started with a 3 min warm-up at 8 m/min, and electric shocks were used sparingly to motivate the rats to run [7]. We chose the treadmill running because the intensity and duration of exercise can easily be controlled, differentially of the voluntary wheel running.

2.2. Methods of protein immunodetection

2.2.1. Tissue preparation

The hippocampal formations of twenty-seven animals from each age group ($n = 9$ in the control, low-intensity exercise and high-intensity exercise subgroups) were removed immediately after decapitation and homogenized in 0.01 M Tris–hydrochloride (pH 7.6) containing 5.8% of sodium chloride, 10% of glycerol, 1% of Nonidet P40 (NP-40), 0.4% of ethylenediamine tetraacetic acid (EDTA) and protease inhibitors. Animals submitted to physical training were killed 1 h after the last exercise session. Samples were sonicated and stored at −80 °C.

2.2.2. Enzyme-linked immunosorbent assay (ELISA)

Hippocampal BDNF levels were examined by ELISA kit E-max® (Promega). Samples previously stored at −80 °C were centrifuged for 5 min at 14,000 rpm at 4 °C and the supernatant transferred to a 96-well plate (Corning Costar) coated with anti-BDNF (1:1000) then incubated for 2 h at room temperature. After this period, the plate was washed with Tris-buffered saline Tween-20 (TBS-T) and incubated with the following antibodies: anti-human (1:500) for 2 h, and conjugate anti-IgY HRP (1:200) for 1 h. Then, color reaction with tetramethyl benzidine was quantified in a plate reader at 450 nm (Quick Elisa).

2.2.3. Immunoassay

Hippocampal levels of inflammatory cytokines were determined by means of immunoassay. Samples previously stored at −80 °C were centrifuged for 5 min at 10,000 × *g* at 4 °C and cytokine concentrations measured with a Millipore multiplex Rat Cytokine Kit on the Luminex® xMAP® platform. The levels of tumor necrosis factor alpha (TNFα), interleukin 6 (IL6) and interleukin 10 (IL10) were determined.

2.3. Histological methods

2.3.1. Tissue preparation

Seventeen animals from each age group ($n = 5$ for control subgroup, $n = 6$ for low-intensity exercise subgroup and $n = 6$ for high-intensity exercise subgroup) were deeply anesthetized (Tionembutal, 50 mg kg, i.p.) and perfused transcardially with solution of 0.01 M phosphate-buffered saline (PBS), followed by solution containing 4% formaldehyde in 0.1 M phosphate-buffered, pH 7.4. Animals submitted to physical training were killed 1 h after the last exercise session. After perfusion, the animals' brains were removed from the skull, cut coronally with a vibratome (Leica) in 50 μm-thick slices and stored at −20 °C in the biological tissue bank in our laboratory (for preservation of tissue). To inhibit the formation of ice crystals that damage the structure of cells, the slices were maintained in an antifreeze solution containing 30% of sucrose, 1% of polyvinylpyrrolidone 40 (PVP-40) and 30% of ethylene glycol in PB (pH 7.2).

2.3.2. Ki-67 immunohistochemistry

The occurrence of hippocampal cell proliferation was investigated by mean of Ki-67 marker. Hippocampal slices previously

stored in the tissue bank were selected in order to analyze the cellular proliferation rate in the hippocampal region of the dentate gyrus (bregma −2.8/−3.6 mm) [19]. Five slices per animal were pretreated with 3% of H_2O_2 for 10 min to block endogenous peroxidase activity, rinsed in PBS, pre-incubated for 45 min in PBS containing 10% of normal serum and 0.2% of Triton X-100, and then incubated in primary antibody against the Ki-67 proliferation marker (1:1000; Abcam) at 4 °C overnight. Paired slices of each subgroup were processed in the same vial in order to minimize the differences during the immunohistochemical procedure. After a sequence of procedures (similar to that described by Gomes da Silva et al. [8]), the slices were finally washed in PBS, mounted on gelatin-coated slides, dehydrated, coverslipped with Entellan (Merk). Afterwards, the hippocampal region of the dentate gyrus of each animal was digitized with a bright-field microscope (Nikon Eclipse 6600) for quantitative analysis. To analyze the Ki-67 staining, we used a similar method to that described by Scorza et al. [21] and Gomes da Silva et al. [8]. Briefly, the significant pixels were then converted into binary matrix (black and white) and quantified by the black pixels sum per area (i.e. density of Ki-67 staining). The quantification of pixels was carried out by software that allows matrix manipulations (Matlab) and in images with the same resolution.

2.3.3. Fluoro-Jade B

A sequence of five hippocampal slices per animal (bregma −2.8/−3.3 mm) [19] previously stored in the tissue bank was selected to observe the immunohistochemical staining of Fluoro-Jade B (FJB), a derivative of fluorescein anionic tribasic that selectively labels degenerating neurons [20]. To perform the FJB histochemical staining, we used a similar method to that described by Gomes da Silva et al. [8]. As a positive control, we added to the immunohistochemical procedure a slice of the hippocampal region (from another project) of an animal injected with 350 mg/kg of pilocarpine (a potent cholinergic agonist that induces *status epilepticus* and leads to severe widespread cell loss in several brain areas). Then, hippocampal slices were rinsed in distilled water, mounted on slides, coverslipped and analyzed qualitatively in confocal microscope (Nikon Eclipse 6600) by two independent investigators.

2.4. Statistical analyses

Statistical analyses were carried out using a SPSS software version 17.0 (SPSS Inc., Chicago, IL). The Shapiro–Wilk and Levene tests were used to verify data normality and homogeneity, respectively. Data with normal distribution or homogeny variances were compared by two-way ANOVA followed by Bonferroni post hoc test. Differences were considered significant when $p < 0.05$. Data with non-normal distribution were compared by nonparametric Kruskal–Wallis test followed by Mann–Whitney tests and Bonferroni correction when $p < 0.05$. Results are presented as mean and standard error of the mean (±SEM).

3. Results

3.1. Hippocampal BDNF levels

We investigated the hippocampal BDNF levels in rats at different stages of postnatal brain development submitted to two physical exercise intensities. Rats submitted to high-intensity exercise from P41 to P50 presented a significant increase of the hippocampal BDNF levels (35.51 ± 3.76 pg/ml) when compared to low-intensity exercise (24.05 ± 1.38 pg/ml; $p = 0.003$) and control rats (26.57 ± 2.75 pg/ml; $p = 0.026$) (Fig. 1). No significant difference was found in other age groups ($p > 0.05$).

3.2. Hippocampal cytokine levels

A significant increase of the hippocampal levels of pro-inflammatory cytokine IL6 was detected in rats submitted to high-intensity exercise from P21 to P30 (36.11 ± 3.79 pg/ml) compared to control rats (5.84 ± 2.10 pg/ml, $p = 0.001$) (Fig. 1). From P41 to P50 period, elevated levels of pro-inflammatory cytokine TNFα were found in the hippocampal formation of rats submitted to high-intensity exercise (TNFα = 15.19 ± 5.50 pg/ml) in relation to their control rats (TNFα = 2.58 ± 0.39 pg/ml, $p = 0.014$). Elevated hippocampal levels of anti-inflammatory cytokine IL10 were also detected in rats submitted to high-intensity exercise from P41 to P50 (1309.68 ± 459.13 pg/ml) compared to their controls (56.72 ± 41.38 pg/ml, $p = 0.009$) (Fig. 1). In other age groups, no significant difference was found in the hippocampal inflammatory cytokine levels ($p > 0.017$; p-value after correction of Bonferroni).

3.3. Cellular proliferation and degeneration in the dentate gyrus

The Ki-67 marker was used to analyze the cellular proliferation rate in the hippocampal formation of developing rats submitted to two exercise intensities. Proliferative cells marked with Ki-67 were observed in subgranular zone and hilar region of the dentate gyrus in all age groups (Fig. 2A). However, quantitative analysis revealed that the density of proliferating cells was significantly higher in the dentate gyrus of rats submitted to low-intensity exercise from P21 to P30 compared with high-intensity exercise and control rats ($p < 0.001$ for both) (Fig. 2B). From P31 to P40, a significant increase of proliferative cell density was found in rats submitted to high-intensity exercise when compared to low-intensity exercise and control rats ($p < 0.001$). In other age groups, no significant difference in the density of proliferating cells was detected among control, low- and high-intensity exercise rats ($p > 0.05$) (Fig. 2B). To verify the possibility of cellular degeneration, we used FJB histochemical staining in the dentate gyrus of rats submitted to two physical exercise intensities at different stages of postnatal brain development. In the positive control, the FJB staining was observed in neurons located in the hilar region of the dentate gyrus (Fig. 3). However, no degenerating neuron was found in the dentate gyrus of control rats or those submitted to two exercise intensities during postnatal brain development (Fig. 3).

4. Discussion

Our study examined the influence of exercise intensity at different stages of postnatal brain development in rats. We found that exercise-induced neuroplastic effects on BDNF levels and cellular proliferation in the hippocampal region are dependents of exercise intensity and developmental period. Moreover, it was noted that exercise intensity is an inflammation-inducing factor and that exercise-induced inflammatory response during postnatal brain development is also related to developmental stage.

BDNF is member of a neurotrophin family known to play a fundamental role during brain maturation and development, such as branching and remodeling of dendrites and axons, functional maturation of excitatory and inhibitory synapses, and cell death by apoptosis [13]. Throughout development, BDNF expression is strictly controlled and its alteration can cause abnormal morphological and functional development of the brain [2,13]. For example, BDNF absence in mice results in learning deficits [9]. In the present study, we detected a significant increase in hippocampal BDNF levels in adolescent rats submitted to high-intensity exercise from P41 to P50. Based on the fact that the BDNF is induced by exercise in adolescent and adult animals [8,12], we expected that physical stimulus in developing rats

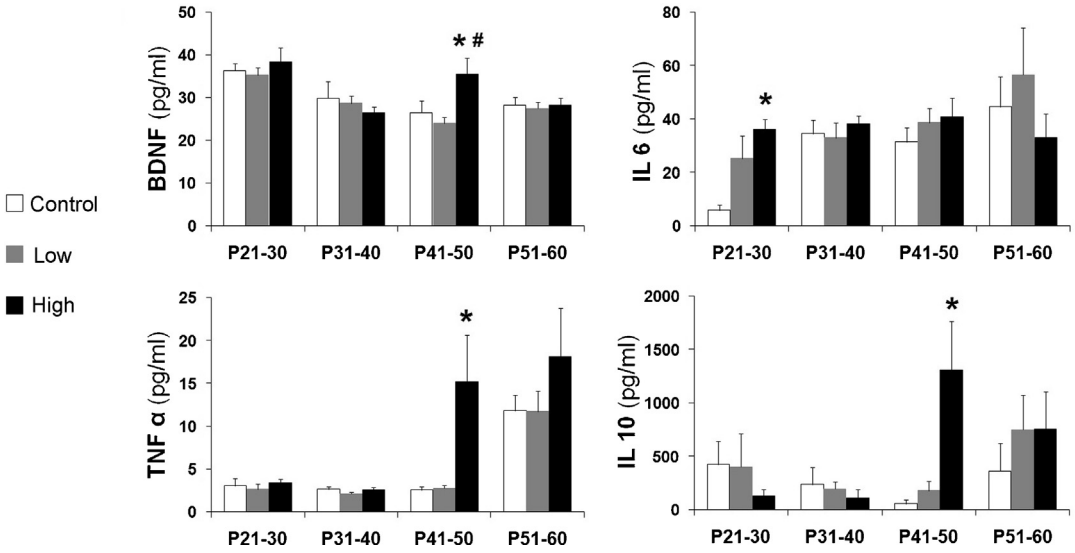

Fig. 1. Hippocampal levels of BDNF and anti- (IL10) and pro- inflammatory (IL6 and TNF-α) cytokines in rats submitted to two exercise intensities (low and high) at different stages of postnatal brain development. $^{*}p < 0.05$ compared to control group; $\#p < 0.05$ compared to respective exercise group. BDNF statistical analysis was performed by two-way ANOVA followed by Bonferroni's post hoc test. Cytokines statistical analyses were conducted by Kruskal–Wallis test followed by Mann–Whitney tests and Bonferroni correction (0.05/3 = 0.017).

could also increase the hippocampal BDNF at different stages of postnatal brain development. However, the results did not show this effect. Moreover, elevated levels of inflammatory cytokines were also found in the hippocampal formation of rats submitted to high-intensity exercise from P41 to P50. Inflammatory cytokines can exert dual effects (positive or negative) on brain functions. These effects are dependent on numerous factors, such as the type of cytokine produced, the functional state and type of stimulated cells, the concentration and the duration of exposure to the cytokines. The over-expression of pro-inflammatory cytokines in the brain may contribute to unsuccessful maintenance of neuronal communication and brain functions. It has been observed that pro-inflammatory cytokines enhance activity of stress-activated protein kinases and impair the brain's ability to maintain hippocampal long-term potentiation (LTP) [18], a physiological process involved in memory consolidation. In opposition, it was demonstrated that inflammation-linked LTP impairment can be reversed by intracerebroventricular infusion of the anti-inflammatory cytokine IL10 [16]. Although of these interesting data reveal an antagonic effect of the anti-inflammatory cytokines on deleterious events produced by pro-inflammatory cytokines, we cannot ensure that our results in rats submitted to high-intensity exercise from P41 to P50 are linked to beneficial neuroplastic effects.

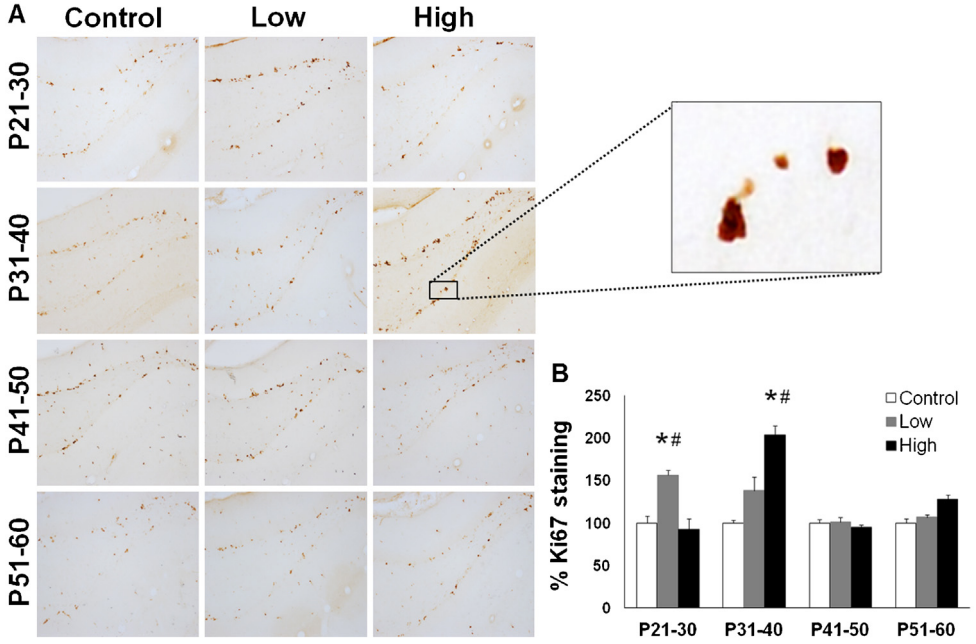

Fig. 2. Cellular proliferation in the dentate gyrus of developing rats submitted to two exercise intensities. Ki67 immunoreactivity (A) and percentage of Ki67 staining (B) at different stages of postnatal brain development of rats submitted to low- and high-intensity of exercise. $^{*}p < 0.05$ compared to control group; $\#p < 0.05$ compared to respective exercise group. Two-way ANOVA followed by Bonferroni's post hoc test. Scale bar = 150 μm.

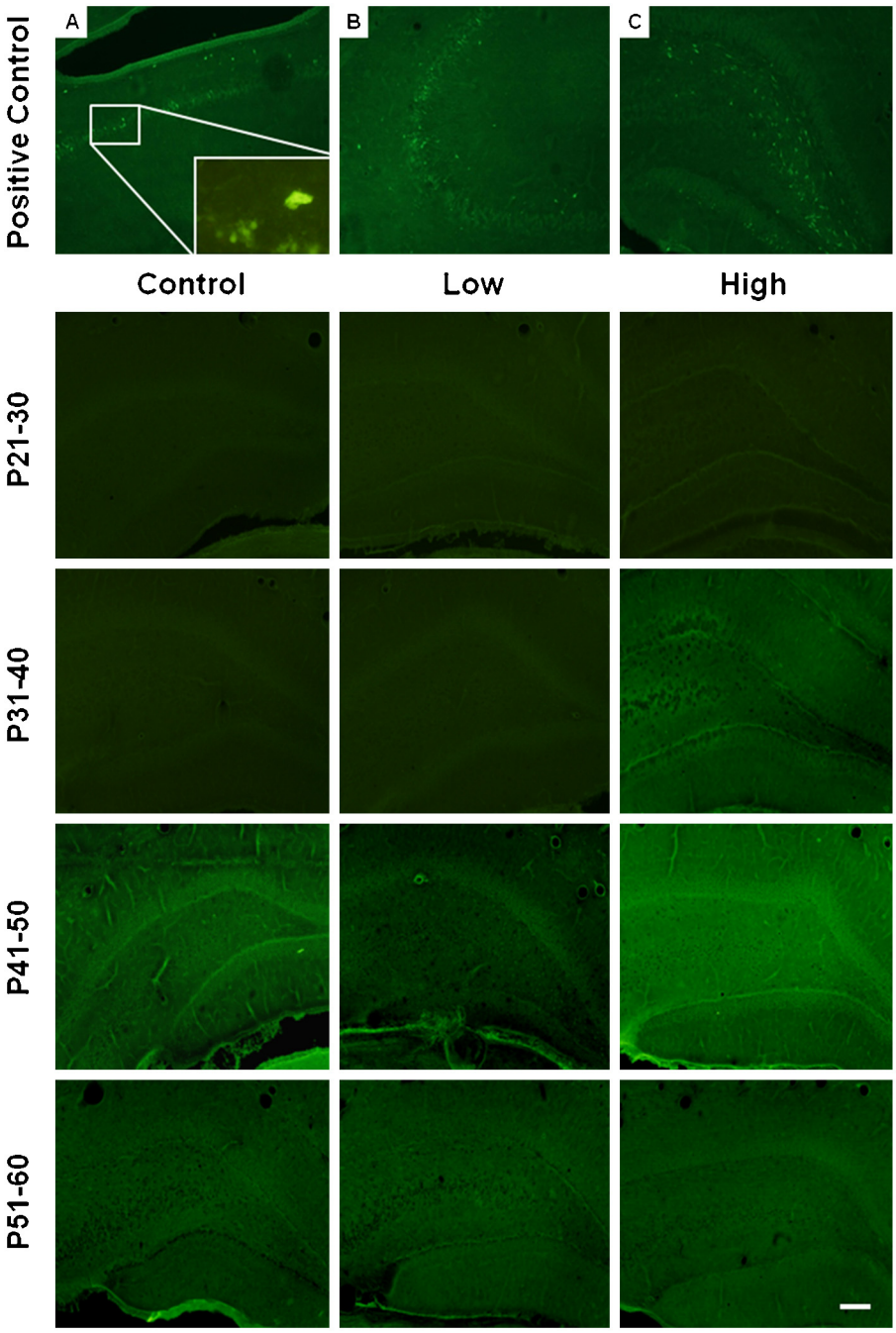

Fig. 3. FJB histochemical staining in the dentate gyrus of developing rats submitted to two exercise intensities. FJB staining in positive control was observed in neurons (fluorescent green) located in the pyramidal cell layer of CA1 (A) and CA3 (B) and in the hilus of the dentate gyrus (C). No degenerating neuron was found in the hippocampal region of developing rats submitted to low- and high-intensity of exercise. To ensure the reliability of results, some samples received a greater time exposure than the other samples. Scale bar = 150 μm.

As mentioned above, over-expression of inflammatory cytokines could result in negative and positive changes for the developing brain. In our study, high-intensity exercise increased both pro- and anti-inflammatory cytokines (TNFα and IL10, respectively) in the hippocampal formation of rats exercised in the P41 to P50 period. Nevertheless, no hippocampal degenerating neuron was found in these animals. A possible explanation for these results could be attributed at least to the exercise-induced BDNF. Indeed, a promising interaction between growth factors and cytokines has been described [17,23]. It has been reported that BDNF administration stimulates IL10 secretion [17] and reduces the inflammation-induced brain lesions extension [23]. Taking into account the interplay between growth factors and cytokines, it is possible that the increase in IL10 levels in our study could also be related to the increase of BDNF levels detected in the hippocampal formation of rats submitted to high-intensity exercise from P41 to P50. Altered neuronal activity and genic expression might be other factors involved in this process.

In the earliest stages of development, positive and negative neurobiological effects in distinct exercise intensities were observed. In the P21 to P30 period, low-intensity exercise increased the cell proliferation rate in the dentate gyrus while high-intensity exercise during the same period induced a significant increase in the hippocampal levels of pro-inflammatory cytokine IL6. These data

support the idea that beneficial effects in the developing brain depend upon low intensities of exercise [15]. On the other hand, the high-, but not the low-, intensity exercise paradigm resulted in a significant increase of proliferative cell density in rats trained from P31 to P40. These findings reveal that new cell formation may vary according to exercise intensity and developmental stage of the brain. It is important to point out that new cell formation induced by exercise in these early stages could have a significant impact on brain structure and functional development. Studies in adult animals have shown that physical exercise enhances cell proliferation and survival in the dentate gyrus and increases the magnitude of hippocampal LTP and improves spatial learning and memory [14,25]. Considering that new cell formation in the hippocampal region is most prevalent in early life [14], we speculate that exercise-induced cellular proliferation in these animals may also be accompanied by improved cognitive capability. Nevertheless, further studies are needed to establish this relationship between exercise intensity, cell proliferation and cognitive functions during postnatal brain development.

In conclusion, the present study has shown that two intensities of exercise may result in positive and negative changes for the developing brain. Depending on the developmental stage, low- or high-intensity of exercise can enhance the cell proliferation rate in the dentate gyrus and increase inflammatory cytokines and neurotrophic factor levels in the hippocampal formation of developing rats. These findings indicate that exercise-induced neuroplastic effects in developing rats depend on both age and training intensity.

Conflict of interest

The authors declare that they have no conflicts of interest.

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